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PAPER CHROMATOGRAPHY OF PLANT VIRUSES

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BOTANY

PAPER CHROMATOGRAPHY OF PLANT VIRUSES [1])

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1. *Introduction*

Relatively little work has been carried out on the development of paper chromatographic methods for identification or isolation of viruses. COCHRAN (1947) using juice of tobacco mosaic diseased Turkish tobacco plants found that tobacco mosaic virus (T.M.V.) moved in water buffered at a pH level of 4.5 or higher in strips of filter paper. He located the position of the virus by applying a reagent which gave a pink colour with the arginine present in the virus. SHEPARD (1952) investigated the behaviour of various members of the seven coliphages on filter paper using water with or without low concentrations of sodium chloride, bovine albumine, or mixtures of both as solvents. Applying the proper solvent, he was able to separate certain types of phages by paper chromatography.

Trying to isolate certain plant viruses by means of a common physical method, viz. differential centrifugation, highly infective virus preparations were obtained by the present authors, which showed, however, no specific particles in electron micrograms as compared with analogous preparations from the sap of healthy plants. As mixtures of related substances with small differences in molecular structure can often be separated with chromatographic techniques, we attempted to get rid of the impurities in the virus preparations by means of paper chromatography. It is known that viruses, e.g. T.M.V. (TAKAHASHI, 1951), strongly absorb ultra-violet light (U.V.) in the neighbourhood of 265 mμ. This fact might be useful to locate the viruses on the paper chromatograms. A method analogous to the one developed by MARKHAM and SMITH (1949) for the identification and estimation of purine and pyrimidine bases from nucleic acids, was applied.

In the present paper an account is given of the methods used for

[1]) This work has been carried out at the Institute for Phytopathological Research at Wageningen.

chromatography of plant viruses on filter paper and of the results of some experiments on the chromatographic properties of T.M.V. in various solvents. The advantages of the method and its limitations are discussed with respect to the results of this investigation.

2. *Materials and methods*

Most of the experiments to be described were carried out with purified T.M.V., only a few with sap from field-grown mosaic diseased tobacco plants. The virus was purified according to the conventional method, i.e. low-speed centrifugation of sap of mosaic diseased tobacco plants that was heated beforehand for a short time at 60° C; the virus was salted out with ammonium sulphate (25 g to 100 ml of clarified sap), resuspended in water and precipitated with acetic acid at its iso-electric point (pH of 3.4). The procedure of salting out and precipitation with acid was repeated several times. The purified virus was stored at 0° C. The nitrogen content of this preparation was 0.13 mg aeq. per ml.

Young leaves of diseased field-grown tobacco plants were crushed with pestil and mortar, squeezed through cheesecloth and centrifuged at 12.000 r.p.m. during half an hour until clear. The clear sap was used in some of the experiments.

Chromatography was carried out on paper strips, 57 cm long and 6 cm wide, cut from sheets of WHATMAN no. 1 filter paper (Fig. 1a) [2]). A sample of 0.09 ml to be chromatographed was added to the paper in a pencil indicated area of 4 cm wide and 3 cm long (called "origin"), situated 6 cm from the top edge of the strip and 1 cm from both side edges. The strip was folded along the line C–D 1.5 cm below the top and this edge was clasped between two glass plates of 6.5 × 6 cm placed in a porcelain embedding dish of 9 × 6 × 1.5 cm. Ten ml of solvent were immediately added to the dish in order to prevent drying of the sample on the strip. When water or salt solutions were used as solvents, the chromatograms were developed in an atmosphere saturated with water. In case of buffer solutions or an acetone-water mixture, the atmosphere was brought into equilibrium with the solvent used. The solvent was allowed to descend over a distance of 44 cm below the area where the sample was added to the strip. This took about four hours at 18°–20° C. The chromatogram, when used for locating the U.V. absorbing substances, was then removed to dry within 20–25 minutes at about 55° C.

The solvents used were twice destilled water, sodium chloride solutions of various concentrations, sodium acetate–acetic acid buffers, according to Walpole, ranging from pH 3.6–6.0 and acetone-water (50 % v/v).

The dried paper strips were cut lengthwise into halves along the line AB

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[2]) The authors are greatly indebted to Mr C. F. SCHEFFEL for the preparation of the photograph, reproduced in Fig. 1 and for many valuable suggestions regarding photography.

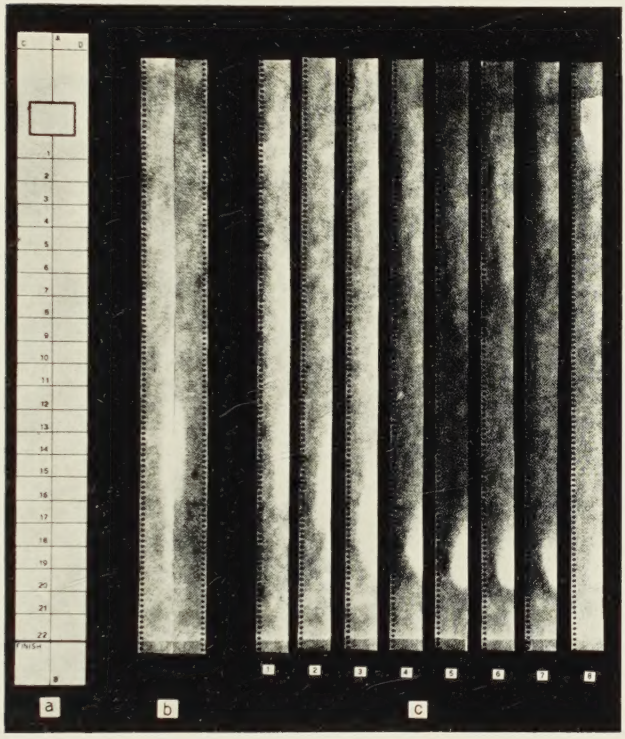

Fig. 1. a. Diagram of a strip of filter paper used in the experiments. b. Photogram of a chromatogram of a purified T.M.V. preparation run with water as solvent. The photogram was made with U.V. of about 265 mμ. c. Series of photograms of chromatograms of a purified T.M.V. preparation run with various solvents, viz. water (1), sodium chloride solutions of 0.017 M (2), 0.1 M (3), 1.0 M (4), 1.5 M (5), 3.1 M (6), 4.3 M (7), and acetate-acetic acid buffer of pH 3.6 (8). (All photographed with light of 265 mμ)

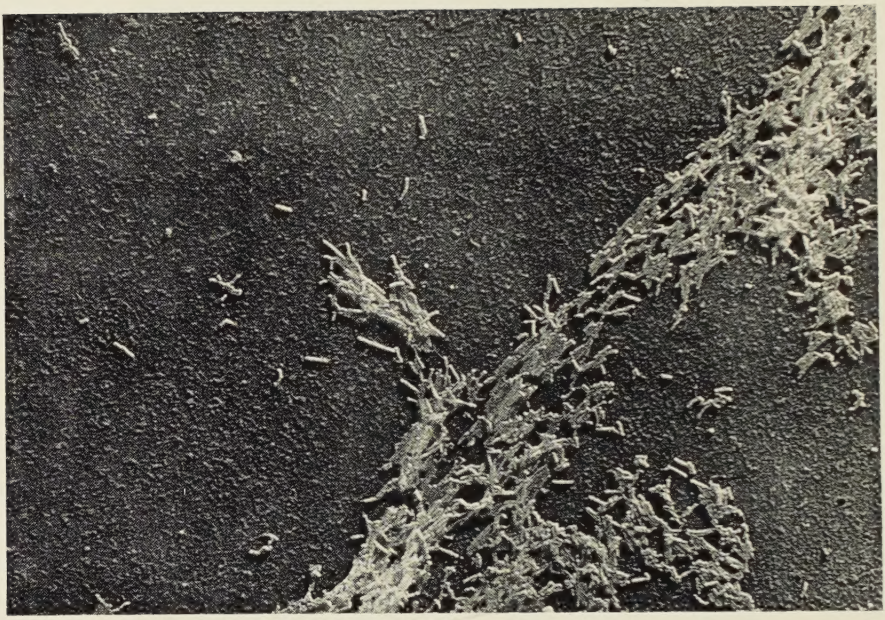

Fig. 2. Electron micrograph of an extract made from a part of a chromatogram obtained from the sap of a mosaic diseased field-grown tobacco plant and developed with water. The magnification is $\times 25{,}125$. (The electron micrograph was made by Miss CHR. VAN DER SCHEER, Laboratory of Physics and Meteorology, Wageningen)

(Fig. 1a). Contact prints (in this paper called photograms) of one half of the strips were made in U.V. of various well-defined wave lengths on 35 mm film bromide photopaper (Crumière normal DN 11) in order to locate the U.V. absorbing substances present on the paper strip. Therefore, the half paper strip was placed over a strip of film paper and fixed on a cylinder of 18 cm diameter which rotated at a speed of 30 r.p.m. in the light produced by the monochromator M 4 Q from a Zeiss-Opton spectrophotometer from which the cuvette holder had been removed [3]). The distance between the surface of the cylinder and the window of the monochromator was 33 cm. The monochromator slit was fully opened which corresponded with a bandwidth of 6.6. mμ at a mean wave length of 265 mμ. Generally, the time of exposure was 7 minutes.

The place on the chromatogram containing the material that absorbed the light of about 265 mμ showed on the photogram as a white or grayish spot. From the unexposed half of the chromatogram the corresponding zones were cut for tests for virus activity. The cut paper pieces were then extracted with distilled water and the extracts inoculated on detached leaves or on leaves which were still on the test plants. To prevent denaturation of the viruses by heating or drying, sometimes two chromatograms of the same object were run in a completely identical way with an interval of 45 minutes. When the solvent on the first chromatogram had reached the finish it was dried and photographed as described. When the second chromatogram was ready, it was cut into pieces while still wet according to the photogram obtained from the first chromatogram. The wet pieces were extracted and the liquids used for electron microscopy or inoculation experiments.

3. *Results*

3.1. Photograms of chromatograms

Fig. 1b shows a picture of a photogram made with U.V. of approximately 265 mμ from a chromatogram of a purified T.M.V. preparation with distilled water as solvent. It shows the presence of a strongly U.V. absorbing spot with an R_F [4]) of 0.62. It is noteworthy that in several chromatograms of the same virus preparation the R_F showed only very slight variations when the chromatograms were run under exactly the same conditions. Strips of filter paper without the virus preparation but run with solvent only did not show any U.V. absorbing spot except a narrow, weak U.V. absorbing zone near the solvent front.

Photograms of a chromatogram of a T.M.V. preparation with water as solvent were produced with U.V. of various wave length bands,

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[3]) The authors gratefully acknowledge the help of Mr E. J. Hagen for the construction of some supplementary instruments.

[4]) $R_F = \frac{\text{distance travelled by spot}}{\text{distance travelled by the liquid front}}$

ranging from 244–250 mμ, 250–256 mμ, 256.8–264 mμ, 261.2–267.8 mμ, 270–279.3 mμ, and 280–290 mμ, respectively. The best photograms were obtained with bands of 256.8–264 mμ and 261.2–267.8 mμ.

In Fig. 1c (no. 1–7) photograms of a number of chromatograms of the same T.M.V. preparation with sodium chloride solution of varying concentrations as solvents, are presented. It is obvious from these photograms that increasing concentrations of sodium chloride up to a concentration of 1.5 M increased the R_F of the strongly U.V. absorbing spot. Higher concentrations than 1.5 M did not result in a further increase. Furthermore, concentrations of 1 M and higher showed a striking reduction of the dimensions of the comet-like spot, particularly with respect to its tail. On the other hand, it is notable that at increasing salt concentrations a more or less U.V. absorbing zone appeared at the origin of the chromatogram.

Regarding the acetate-acetic acid buffer solutions of pH 3.6, 4.0, 4.6, 5.0, 5.6, and 6.0, respectively, it was observed that a weak U.V. absorbing spot with an R_F of 0.85 was present. Moreover, the photograms showed by decreasing pH value an increasing U.V. absorption in the origin and in the zone directly below the origin (Fig. 1c, no. 8). Finally, the U.V. absorbing front zone appeared to become more distinct, especially with the buffer solution of pH 4.6 and higher.

Chromatograms of the T.M.V. preparation were also run with an acetone-water mixture. The R_F of the strongly U.V. absorbing spot remained the same as in the chromatograms developed with water but the spot was nearly 1.5 times longer.

Clarified sap of the younger leaves obtained from field-grown mosaic diseased tobacco plants developed with water as solvent gave a chromatogram with a highly U.V. absorbing spot in the same position as in the case of the purified T.M.V. preparation. No chromatograms from sap of comparable healthy tobacco were made owing to lack of healthy, field-grown plants. However, sap obtained from young healthy tobacco plants grown in the greenhouse, did not show the same U.V. absorbing spot as known from the T.M.V. preparation.

3.2. Infection experiments

Chromatograms prepared from the T.M.V. preparation and developed with water contained most of the T.M.V. in the U.V. absorbing spot described in section 3.1. Between the solvent front and the U.V. absorbing spot no virus could be detected whereas between this spot and the origin lower quantities of virus than in the spot itself could be recovered. In the chromatograms obtained from clarified sap of field-grown tobacco plants with water, T.M.V. could be detected by infectivity tests only in the area of the U.V. absorbing spot.

Infectivity tests with extracts from parts of the chromatograms of the T.M.V. preparation developed with various sodium chloride concentra-

tions revealed that the strongly U.V. absorbing spots represented in the photograms of Fig. 1c did not contain T.M.V. when salt concentrations of 0.1 M or higher were used but that the virus was present in the origin of the chromatogram and in the neighbouring zone whose length tended to decrease with increasing salt concentration.

With acetate-acetic acid buffer of pH 3.6 the virus remained localized at the origin of the chromatogram. At pH values of 4.0, 4.6, 5.0, 5.6 and 6.0, virus could also be recovered from zones neighbouring the origin but in these cases the zone where the virus was present was never identical with the U.V. absorbing spot on the lower part of the chromatogram.

In the chromatogram developed with acetone-water 50 % (v/v) the zone with the highest virus activity covered completely the elongated, strongly U.V. absorbing area.

In all the infectivity tests, extracts from zones of the dried chromatograms gave rise to the formation of less local lesions on *N. glutinosa* leaves than extracts from the corresponding zones of the comparable wet chromatograms.

4. *Discussion*

As data in virus literature indicate that purified T.M.V. strongly absorbs U.V. of 265 mμ, it seemed reasonable to assume that the distinct U.V. absorbing spot in the chromatograms of the used T.M.V. preparation could be identified as T.M.V. itself, especially since in the chromatograms developed with water or acetone-water zones of strong U.V. absorption covered those of high virus activity.

Electron micrograms (Fig. 2) [5]) made from extracts from U.V. absorbing zones of chromatograms of sap of field-grown tobacco plants and run with water, suggested this also as only these zones contained rod-shaped particles which resembled those known from T.M.V. It is interesting that these particles occurred in clusters. It should be remarked that the preparation used for obtaining the published electron micrograph was made from the photographed half of a dried chromatogram and thus exposed to U.V. of 265 mμ.

Using sodium chloride solutions or acetate buffers as solvents, however, it was found that there was no direct relationship between the occurrence of virus activity and the presence of material able to absorb strongly U.V. as shown by the photograms. Sodium chloride solutions used as solvents did not reduce the transmission of U.V. but made the paper more penetrable as can be seen in Fig. 1c no. 4–7. In contrast to this, ammonium sulphate and ammonium chloride proved to be fully

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[5]) The authors are greatly indebted to Miss CHR. VAN DER SCHEER, phys. dra, electron-microscopist at the Laboratory of Physics and Meteorology, Wageningen, for making the electron microscopic examinations and for the electron micrograph reproduced in Fig. 2.

impenetrable. The same holds, although to a lesser extent for citric acid when used in citric acid-phosphate buffers.

Apparently, T.M.V. has the same R_F in water and acetone-water mixture as a substance (or group of substances) still present in the purified virus preparation which is (are) able to absorb strongly U.V. of 265 mμ. Using, however, salt solutions the virus remains more or less in the region where it was applied to the paper strip, apparently as a consequence of salting out absorption on filter paper (LEDERER and LEDERER, 1954). With buffer solutions of a pH near the iso-electric point of 3.4, the virus remains also more or less immobile. Also at higher pH values the virus tends to remain near the origin of the chromatogram, which might be explained by supposing a secondary effect of the buffer ingredients as salting out reagents.

It is not impossible that under certain conditions of salt or pH the concentration of T.M.V. in a special zone becomes so high that the virus nucleoprotein is responsible for the weak U.V. absorption which can be noticed on the photograms (Fig. 1c, no. 5, 6, 7) in the region of the origin and neighbouring zones of the chromatograms.

It was noticed in infection experiments that T.M.V. is confined to a smaller area on the chromatograms of sap from field-grown tobacco plants than on the chromatograms of a purified virus preparation. This might be due to the presence of substances (perhaps proteins?) in the sap which are lacking in the virus preparation or occur in smaller concentrations. This might be suggested by the experience of SHEPARD (1952) who added albumine to the solvent to obtain a better separation of different phages in paper chromatograms.

The reproduced electron microgram shows that paper chromatography of low-speed centrifuged sap of diseased tobacco plants results in fairly pure preparations for use in electron microscopy.

Chromatography on filter paper using contact photography in U.V. of specific wave lengths as indication method might also find application in the study of strongly U.V. absorbing substances occurring in the normal cell.

5. *Summary*

A description is given of some experiments on the chromatographic behaviour of T.M.V. on filter paper strips with water, various sodium chloride concentrations, acetate-acetic acid buffer solutions, and an acetone-water mixture, respectively, as solvents. Attempts to identify the presence of T.M.V. with a spot that strongly absorbed U.V. of about 265 mμ wave length in the chromatograms were unsuccessful. It has thus far been possible to locate the virus only by inoculating *Nicotiana glutinosa* with extracts prepared from zones cut from the paper strips. The method proved to be promising as a simple test for the estimation of certain impurities in purified virus preparations and for the preparation of viruses for electron microscopy from low-speed centrifuged sap of diseased plants.

REFERENCES

COCHRAN, G. W., A chromatographic method for the detection of tobacco-mosaic virus in juice from diseased Turkish tobacco plants. Phytopath. **37**, 850–851 (1947).

LEDERER, L. and M. LEDERER, Chromatography. p. 228 (Elsevier Publ. Co. Amsterdam, Houston, London, New York, 1954).

MARKHAM, R. and J. D. SMITH, Chromatographic studies of nucleic acids. 1. A technique for the identification and estimation of purine and pyrimidine bases, nucleosides and related substances. Biochem. J. **45**, 294–298 (1949).

SHEPARD, C. C., On the chromatographic behavior of the T coliphages in paper strips. J. Immunol. **68**, 179–184 (1952).

TAKAHASHI, W. N., Ultraviolet absorption as a measure of tobacco-mosaic virus nucleoprotein. Phytopath. **41**, 142–145 (1951).